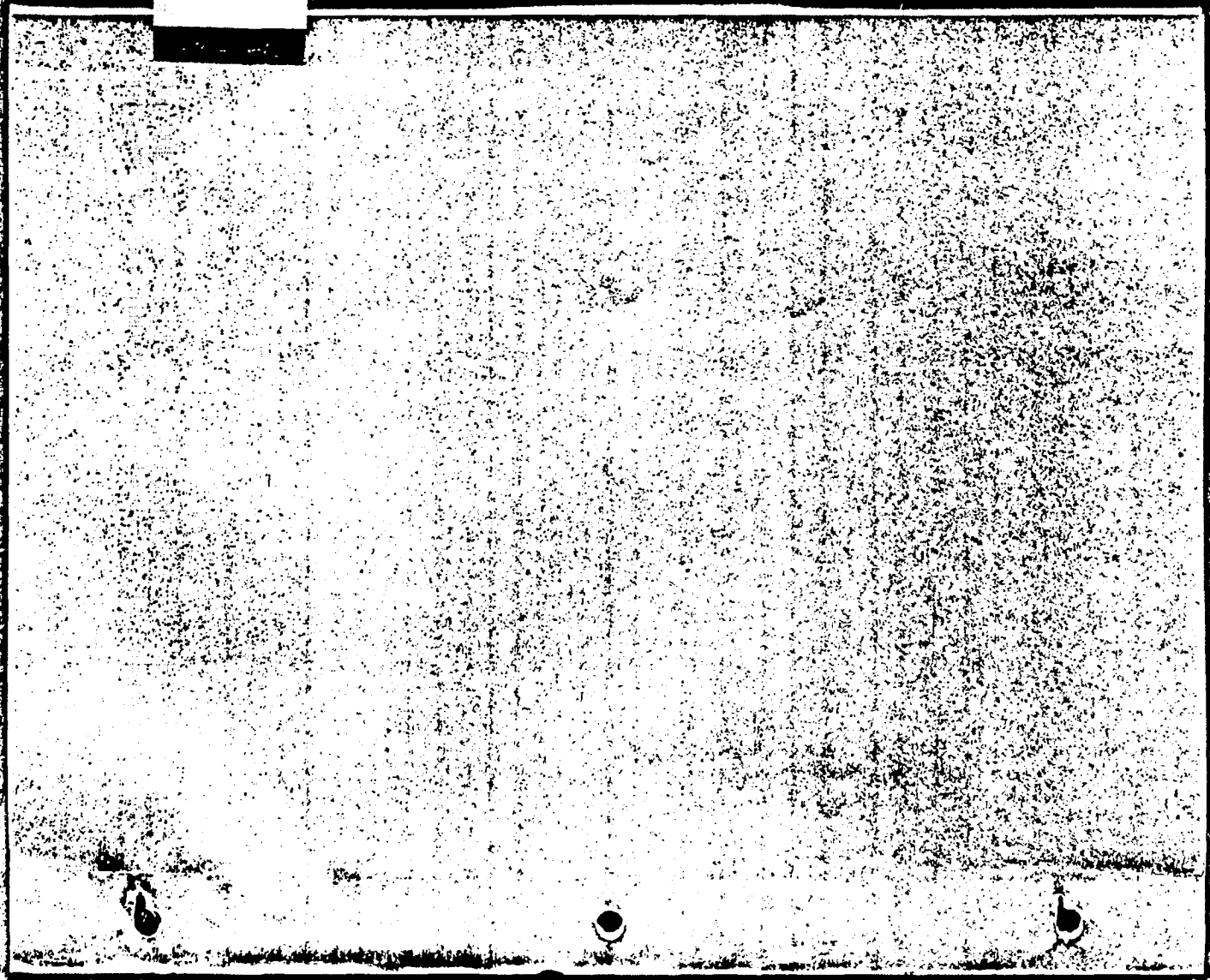

RESEARCH CONTRACT PROPOSAL
(Renewal of Contract No. 0025)

for the period

January 1, 1976 through December 31, 1976

TITLE: HUMAN AHH STUDIES

TO: Dr. John H. Kreisher
The Council for Tobacco Research
110 East 59th Street
New York, New York 10022

FROM: Microbiological Associates, A Division of
Dynasciences Corporation
4733 Bethesda Avenue
Bethesda, Maryland 20014

DATE: August 22, 1975

Vincent L. Ruwet
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Vice President, Contracts
and Administration

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CONTENTS

I. INTRODUCTION

The determination of the relative risks of particular human populations to cancer in general, and tobacco-associated cancers, in particular, is a major goal in the nationwide program to control cancer in man. During the past year, major effort has been directed toward understanding the role of the enzyme complex, aryl hydrocarbon hydroxylase (AHH), in human cancer - for the level of these enzymes seem to be correlated with susceptibility to cancer in model animal systems, and preliminary evidence suggests the same relationship may hold for the human system as well. The confirmation of this latter relationship may be one of the most important studies presently being undertaken in the field of cancer research.

The confirmation of this relationship has been plagued by problems with the reproducibility of the assay system itself. It is proposed in this study that a reproducible assay can be achieved and that this assay be used to establish the relationship between levels of AHH activity and at least eight different cancers, including cigarette smoke-associated lung carcinomas.

II. BACKGROUND

Aryl hydrocarbon hydroxylase (AHH) is the name given to one of the multi-component microsomal-bound enzymes (1, 2). The actual steps involved in this oxidative metabolism are unknown; however, the best guess is that the substrate combines with the oxidized form of a carbon monoxide sensitive hemoprotein called cytochrome P-450. The substrate-cytochrome P-450 complex is then reduced by an electron donated by NADPH-cytochrome c reductase to form a reduced substrate-cytochrome P-450 complex. This complex in turn reacts with molecular oxygen to form a reduced substrate-cytochrome P-450-O_2 complex. A second electron is then added to this complex to yield an active oxygen intermediate that decomposes with the formation of the product and the oxidized P-450. The product of this reaction, if polycyclic aromatic hydrocarbons are substrates, are probably epoxides (3, 4). These intermediates then (a) rearrange spontaneously to form phenols, (b) are enzymatically metabolized to the dihydrodiol via the enzyme epoxide hydrase, or (c) are enzymatically conjugated with glutathione using the enzyme glutathione conjugase.

These enzymes have two properties which make them uniquely important to the study of chemical carcinogenesis. First, metabolism of many substrates (especially PAH) does not necessarily result in detoxification, but rather are converted to water-soluble forms via transient chemically-reactive intermediates that are both cytotoxic (5, 7) and carcinogenic (8-10). Second, that this enzyme system is inducible by certain substrates and that this induction results in the enhanced metabolism of many foreign compounds (11). This latter property is important because, if these metabolic pathways for PAH are etiologically important in the initiation of chemical carcinogenesis, any marked changes in rates of formation of the water-soluble metabolites (e.g. epoxides, phenols, dihydrodiols and glutathione conjugates) or covalently bound metabolites should affect the host's susceptibility to cancer.

In the house mouse Mus musculus, not only is AHH induced by treatment with certain substrates, but also this inducibility is host gene regulated. Treatment with phenobarbital (PB) increases the metabolism of most of drug substrates and in every strain of mouse tested (12). Treatment with 3-methylcholanthrene (MCA) increases the metabolism of very few substrates and in only particular strains (13). These differences probably result from the fact that PB causes a rapid non-specific proliferation of constitutive AHH (14), while MCA induces a new spectrally distinct cytochrome called P^1-450 (15) or P-448 (16) which has different substrate specificities. The ability to respond to

MCA (but not PB) segregates as a single autosomal gene in crosses involving the C57BL/6 (B6) and DBA/2 (D2) strains of mice (13, 17-19). Thomas et al (13) and Kouri et al (20, 21) propose this locus be designated Ah; the allele carried by the B6 mouse (inducible) is Ah^b, and the allele carried by the D2 mouse (noninducible) is Ah^d. Following treatment with MCA, the difference between the AHH levels in various tissues of Ah^b/Ah^b or Ah^b/Ah^d mice are 2-80 fold greater than that of Ah^d/Ah^d animals (17-21). Thus, one can evaluate tumor susceptibility among litter-mates in which the presence or absence of AHH induction is expressed in their tissues. With the use of such a model, other non-specific strain differences - such as characteristic mouse strain differences involving immunology, latent viral infections. nutrition, hormones, stress, or levels of other enzymes - will be theoretically cancelled.

Using this model system, Kouri et al (20-22) have reported that segregants carrying the Ah^b allele are approximately 12 times more sensitive to MCA induced fibrosarcomas than animals homozygous for the Ah^d allele. Thus, it seems likely that the types of metabolites, or just the quantity of these metabolites determined by this novel "inducible" enzyme play a major role in determining the susceptibility of mice to chemical carcinogenesis.

When studying the mixed-function oxidases in man, two major problems become apparent: the availability of tissue and the availability of sensitive enough assay procedures. Human tissues that have been used include cultured cells in vitro (23, 24), placenta (25-27), foreskin (28), biopsied liver (29-31), skin (32), pulmonary (33, 34), and blood macrophages (35), and peripheral blood lymphocytes (36-38). The available assays include: a) measurement of conversion of BP to 3-OHBP spectro-photofluorometrically (39, 40), b) measurement of conversion of BP to water-soluble forms (24, 41), c) quantitive measurement of conversion of BP to its various metabolites using thin layer chromatography (42-44), and d) cytochemical analysis of individual cells by microfluorometry (45-49), and e) direct quantitation of the P-448 or P-450 cytochromes (30, 50, 51, 52). Only a few investigators have reported data concerning the metabolism of actual chemical carcinogens by human tissue. Kuntzman et al (29) reported that benzo(a)pyrene (BP) was metabolized by autopsied human liver microsomes at a rate similar to that observed in rat liver. Merrill and Campbell (31) reported that aflatoxin was also metabolized by human liver obtained at autopsy. Using cultured human cells, both Huberman and Sachs (23) and Kouri et al (24) have recently shown that these cells can metabolize BP to water-soluble forms, and the rate of metabolizing is influenced by the relative number of epithelial-like cells and perhaps by the age of the cells in culture (number of generations). Recently Bast et al (53) reported that peripheral blood monocytes possessed fairly high AHH activities.

The level of AHH can also be influenced by cigarette smoke. Placental tissue from women who smoke contains much more AHH activity than the tissue from non-smoking women (14, 15).

The system which seems to show the greatest promise in determining the role of AHH in human cancer is the mitogen-activated peripheral lymphocyte. Using this system, Busbee et al (37) and Whitlock et al (36) report that AHH can be detected in both fresh and cultured lymphocytes. Moreover, the spectrum of BP metabolites observed is very similar to that observed from the enzyme in rat liver (52, 54, 55). The extent of inducibility seems variable in the human population (56), and most importantly, these differences may be genetically regulated by an autosomal co-dominant allele (58). The AHH response using other inducers, such as 2, 3, 7, 8-tetrachlorodibenzo-(p)dioxin, follow this same regulation (38). Moreover, the activity in human lymphocytes may, in fact, be correlated with hepato oxidation rates (57).

The recent work by Kellermann et al suggests that this genetically regulated level of AHH may influence susceptibility to cigarette-smoke associated lung cancers, just like genetically-regulated levels of AHH effect susceptibility of MCA-induced tumors in mice (21). Kellermann et al (59) reported that a much higher percentage of medically verified lung cancer patients were either high or intermediate AHH inducers; suggesting that not only are polycyclic aromatic hydrocarbons important causes of cancer in humans, but also, that, as in mice, those individuals with a heightened ability to metabolize these chemicals may be more susceptible to the chemically-induced or "spontaneous" cancers. The confirmation of these studies is now being completed in various laboratories.

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III. EXPERIMENTAL

A. From September 1975 to December 31, 1975

1. Establish standard culture conditions

Studies will continue in determining those conditions yielding the most reproducible mitogen-activation of human lymphocytes. Work will center on evaluating the use of purified mitogens (e.g. leucoagglutinin, Pharmacia Fine Chemicals, New Market, N. J.), non-carcinogenic inducers (e.g. 5, 6-benzoflavone), and particular lots of fetal calf serum as ways to standardize mitogen-activation.

2. Establish methods to correct day-to-day fluctuations in mitogen-activation

Even under the most exact culture conditions, differences in rates of mitogen activation may be observed. This probably results from inherent differences in lymphocyte populations in vivo. Whatever the reason, methods may have to be established to correct these day-to-day variations. It is proposed to continue evaluating rates of DNA, RNA and protein synthesis as a method to correct variations in mitogen activation.

3. Studies with the "standard" lot of FCS from GIBCO

It is proposed to determine the reason why cells cultured in the same FCS, but in different laboratories, give different levels of AHH. In collaboration with Dr. T. Yamauchi (M.D. Anderson Hospital and Tumor Institute, Houston, Texas), blood samples from individuals within our laboratories will be exchanged, activated in both laboratories, and cells will be assayed in both laboratories. Frozen mitogen-activated, induced and non-induced samples will be assayed. Determinations of the factor(s) that cause these disparate levels of AHH activity should go a long way in achieving a more standardized assay system.

4. Determination of standard parameters on which to base AHH activity

Six males (from within our own laboratories) will be assayed once a week for three consecutive weeks. The rates of AHH per ug DNA, per rate of DNA synthesis, and per flask will be evaluated. Also, the total amount of AHH produced in response to co-treatment with mitogens and inducers will be determine. Total

activity will be measured at 48, 72, 96 and 120 hours of mitogen and inducer treatment. The use of rates of incorporation of H^3-thymidine in acid precipitable material as a potential correction factor for variation in day-to-day mitogen activation will also be determined at this time.

A second set of six males will be assayed once a week for three consecutive weeks for observations on the use of levels of NADH-dependent cytochome c reductase as standard parameter on which to base AHH activity. As shown in the progress report, the level of this enzyme seems dependent on the amount of mitogen activation (or amount of microsomes) and independent of treatment with AHH inducers. Thus, the level of this enzyme could be a way to obviate the need for counting cells, quantitating DNA, or determining correction factors for mitogen activation. Preliminary work designed to establish the optimal conditions for measuring this enzyme activity will be done in collaboration with Dr. R. Prough (Southwest Medical School, Dallas, Texas).

5. Determination of relationship between fluorometric and radiometric assay for AHH activity

In collaboration with Dr. H. Guirgis (Creighton University, Omaha, Nebraska), the level of AHH in ten standard individuals in our laboratories will be simultaneously studied by both the radiometric assay and fluorometric assay for AHH activity. The radiometric assay to be used by Creighton University involves the quantitation of the rates of metabolism of H^3-benzo(a)pyrene from an organic-soluble to water-soluble forms by mitogen-activated human lymphocytes. The fluorometric assay will be based on the results of the experiments outlined in the previous sections. The exact same culture conditions, including medium, FCS, mitogens, and inducers, will be used in both laboratories.

B. From January 1, 1976 to December 31, 1976

1. Updating assay procedures

Work will continue on evaluating alterations or additions to any facet of the assay procedure which may allow for a more reproducible, more rapid, or more efficient assay of AHH activity. This will include evaluating a) use of ethoxyresorufin as an alternate substrate for AHH activity (see progress report); b) use of alternate assay procedures, such as quantitation of levels of cytochromes in human lymphocytes; c) use of combination of inducers, such as those that specifically alter constitutive, or P-450 mediated, enzyme activity (e.g. tryptamine, B-estradiol, phenobarbital, aminophylline or theophylline), and those that

specifically alter "induced," or P-448 mediated, enzymes (e.g. MCA, 5, 6-benzoflavone, diphenyloxazole, 5-bromobenzanthracene, etc.), and d) new culture conditions, such as novel purified mitogens, chemically-defined medium, and conditions allowing for preferential activations of specific subpopulations of human lymphocytes.

2. Participation in blind protocol

In collaboration with another laboratory (e.g., Creighton University of USC Medical Center, Los Angeles, Calif.), samples of diluted whole blood (or isolated lymphocytes) will be shipped under code to our laboratory and assayed for AHH activity. Samples will be sent every week for a total of four weeks. These samples will include not only multiple samples from the same individual on the same day, but also samples from the same individual on different days. The code will then be broken and results assessed. At the discretion of the Project Officer, the blind assay will either be repeated or studies into the determination of enzyme levels in cancer patients initiated.

3. Determination of enzyme activity in cancer patients

a. Studies on mitogen-activation of lymphocytes from cancer patients

Preliminary studies will center on the problem of mitogen-activating lymphocytes from cancer patients. It is a well-known fact that lymphocytes from cancer patients respond very poorly to mitogens. It is felt that with just minor modifications of the culture conditions that resulted in optimal growth of lymphocytes from non-cancer patients, that some activation of lymphocytes from cancer patients will be observed. A blind assay, utilizing only cancer patients, may be required. The ability of such a blind protocol will be evaluated at that time.

b. Relationship of levels of AHH activity to cancer susceptibility

These proposed procedures are very similar to those originally proposed for this contract, but because of assay difficulties, could not be completed. The population of Menck et al is a good example of the population that will be used because it appears to fulfill the criteria of accessibility as well as availability of adequate numbers of patients. The intial study will be concentrated on the lung cancer population. Fifty to 100 lung cancer patients, 50 to 100 hospital controls and 50 to 100 non-hospitalized controls will be assayed.

Assays will be done at MA at a rate of 28 to 50 per week. This study will also entail a limited questionnaire involvement: including history of cigarette smoking, drug exposure, occupation, etc. Medical verification of the pathological lesion will also be done.

The suggested approach will concentrate on the importance of cancer association and AHH levels. A matched population would seem to be the best obtainable control at this level. The requirement for detailed questionnaire and multivariant analysis of same must be incorporated to observe correlates and associations. Smoking habits, while normally considered a tertiary study variable, are included in this secondary study, since this variable has particular interest to the granting agency. The procedure for this secondary study will include one hundred patients for each of seven cancer types (total 700) and 700 to 800 controls matched for age, sex, ethnic group and smoking habits. The routine fluorometric assay discussed before will be used. However, concurrent preparations for automated analysis will be included in preparation for the tertiary level studies. The questionnaire for this study will be detailed and organized for computerization. This in-depth level of inquiry would give operational experience and possible leads for specific in-depth tertiary studies. This detailed questionnaire is compatible with the 1,500 patients projected. Areas to be emphasized in this secondary level include: (1) normal medical history; (2) detailed employment history - with master breakdown code of probable PAH, etc. exposure; (3) detailed drug history, weighted for those drugs involved in AHH; (4) detailed smoking history; (5) detailed alcoholic consumption history; (6) other variables to be defined - possible psychological evaluations.

The questionnaire validity will be important. Analysis should be undertaken for possible correlations as the data accumulates. These correlations can be used to predict, direct, and avoid unnecessary duplication in the following studies.

IV. PERSONNEL

A. Dr. R. E. Kouri (Principal Investigator) (10%)
(see original proposal)

B. Dr. Richard L. Imblum (Assistant Project Director)
(40%)

Dr. Imblum received his Ph. D. degree in biochemistry from Purdue University. His doctoral research involved studies on a microsomal enzyme, HMG-CoA reductase, in rat liver and Neurospora crassa. He was a postdoctoral fellow of the American Cancer Society at the University of Virginia where his studies concerned the biochemical processes involved in the replication of vesicular stomatitis virus.

Dr. Imblum has had extensive experience in culturing mammalian cells, general enzymology, and mammalian molecular macromolecules including phenol extraction, column chromatography, rate zonal and equilibrium density, ultracentrifugation, polyacrylamide gel electrophoresis of protein and nucleic acids, thin-layer chromatography, paper electrophoresis, and autoradiography.

A copy of Dr. Imblum's curriculum vitae is attached.

Soc. Sec. No. 521-42-5682

CURRICULUM VITAE - RICHARD L. IMBLUM

BIRTH: REDACTED

EDUCATION:

1972	Ph. D.,	Biochemistry Purdue University Lafayette, Indiana
1966	M. S.,	Biochemistry The George Washington University Washington, D. C.
1962	B. A.,	Chemistry University of Colorado, Boulder

PROFESSIONAL AFFILIATIONS: American Society for Microbiology
American Association for the Advancement of Science

PRESENT POSITION: 1975 - present REDACTED

POSITION DESCRIPTION: Contract with the National Cancer Institute, Standardization of Aryl Hydrocarbon Hydroxylase Assay, as a Screening Method to Determine Smoking Hazards in Man; Contract with The Council for Tobacco Research, The Role of Aryl Hydrocarbon Hydroxylase in Human Cancers.

PRIOR EXPERIENCE: 1972 - 1975 REDACTED

1968 - 1972 REDACTED

1967 - 1968

REDACTED

1962 - 1967

REDACTED

PUBLICATIONS - RICHARD L. IMBLUM

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V. BUDGET

A.	Total Direct Labor (See Schedule A)	$ 27,138
B.	Overhead (115% of A)	31,209
C.	Other Direct Costs (See Schedule B)	15,700
D.	Travel ($500/professional plus one trip Los Angeles - Washington, D.C.)	1,500
E.	Total (A - D)	75,547
F.	G & A (16% of E)	12,088
G.	Total Costs	87,635
H.	Fixed Fee (10%)	9,737
I.	Total Cost Before Equipment	97,372
J.	Equipment (See Schedule C)	9,700
K.	Total Cost	$107,072

SCHEDULE A - TOTAL DIRECT LABOR

Name	Function	Time on Project	Total Hours*	$ Hour	$
R.E. Kouri, Ph.D.	Project Director	10%	193	REDACTED	
R. Imblum	Assistant Project Director	40%	770	REDACTED	
C. McKinney	Senior Technician	100%	1,926	REDACTED	
R. Sosnowski	Technician	100%	1,926	REDACTED	
	Total Hours		4,815		
	Total Direct Labor				25,602
	+ 6% Raise				1,536
	TOTAL DIRECT LABOR				$27,138

SCHEDULE B - OTHER DIRECT COSTS

Materials:

Media	$3,000	
Blood samples	500	
Chemicals	5,500	$9,000

Expendable Supplies:

Glassware (production and reuseable)	2,700	
Disposable glassware	4,000	6,700

TOTAL OTHER DIRECT COSTS $15,700

SCHEDULE C - EQUIPMENT

Scintillation Counter, LS 100, 3 Channel, 60 Hz.	$9,500
Source: Beckman Instruments, Inc.	
Plus 2% Sales Tax.....	190
TOTAL EQUIPMENT	$9,690
Amount per Budget	$9,700